J Sci Food Agric 85:418–424 (2005)
DOI: 10.1002/jsfa.1999

Application of transglutaminase to derivatize proteins: 1. Studies on soluble proteins and preliminary results on wool†‡

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Abstract: The use of enzymes in chemical processing is gaining favour due to the reduction of hazardous chemicals and because it is considered to be environmentally safe. The acyl transfer reaction between primary amines and glutamine residues in proteins is catalysed by the enzyme transglutaminase. The efficiency of microbial transglutaminase to attach functional amines and catalyse inter- and intra-molecular crosslinks was investigated using reduced carboxymethylated κ-casein, gelatin and wool. Model systems used in this research gave evidence of both cross-linking of the protein and covalent binding of the primary amine *o*-phosphorylethanolamine to the protein. These data agree with earlier publications that show transglutaminase catalyses the formation of covalent cross-links between the γ-carboxyamide group of glutamine and the ε-amino group of lysine and also the incorporation of primary amines into proteins. Preliminary analysis of treated wool indicated the covalent bonding of the functional amine to the protein. Our goal is to increase the value of wool by enzymatic addition of functional groups to the wool fibre.
Published in 2004 for SCI by John Wiley & Sons, Ltd.

Keywords: *o*-phosphorylethanolamine; transglutaminase; gelatin Blooms

INTRODUCTION

The use of enzymes in the processing of agricultural commodities has steadily increased. Enzymes are used in numerous ways to improve functional properties of foods and fibres.[1] The use of enzymes in the processing of agricultural products is viewed as an environmentally safe procedure when the same results are achieved without exposure to harsh chemicals. Enzymatic approaches have been used to improve the properties of wool, gelatin and collagen hydrolysate as inexpensive and environmentally safe methods.[2,3] The field of industrial biocatalysis was recently reviewed by Zaks.[4] The use of enzymes in the textile industry is well known and accepted; the applications and technologies are many and varied.[5,6]

In the textile industry, enzymes are used in the biofinishing of cotton/wool blends.[3] Protein–glutamine, amine γ-glutamyl-transferase (EC 2.3.2.13), commonly known as transglutaminase (TGase), is an enzyme that catalyses the acyl transfer reaction in which a γ-carboxyamide group of a peptide-bound glutamine (Gln) residue is an acyl donor. The acyl receptor is a primary amine group, usually the ε-amino group of a lysine (Lys) residue;[7–9] *in vivo* this results in the modification of proteins through either intra- or inter-molecular crosslinking. *In vitro* the enzyme also catalyses the covalent attachment of primary amines to Gln residues,[10,11] as illustrated in Fig 1. Mammalian TGases are calcium-dependent.[12] As a class, TGases from various sources display varying reactivity towards Gln residues.[12] Microbial TGase, which was used in this study, differs from the mammalian enzyme in that it is calcium-independent, has a smaller molecular weight and is commercially available.[13] Claims that TGase may improve the quality of wool as in felting, whitening, handling and shrinking have been made.[14] It has been reported that the ability to incorporate various functional groups into the glutamine residues of protein using TGase would be very useful[15] as it might improve and enhance the end use of the protein. In this study we report the incorporation of a primary

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‡Mention of brand or firm names does not constitute an endorsement by the US Department of Agriculture over others of similar nature not mentioned.
(*Received 14 October 2003; revised version received 2 April 2004; accepted 23 June 2004*)
Published online 8 November 2004

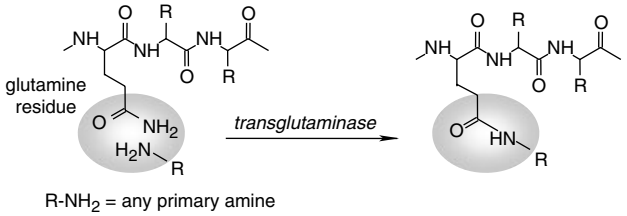

Figure 1. Transglutaminase-catalysed reaction of glutamine residues with primary amines.

amine, *o*-phosphorylethanolamine, into protein–Gln residues. Our overall goal is to be able to add various functional groups onto the Gln residues on wool fibre using TGase. RCM-κ-casein is a protein substrate for TGases.[16] Here we used reduced carboxymethylated (RCM-)κ-casein and gelatin as model systems with TGase to cross-link and/or covalently add a primary amine to the model proteins.

MATERIALS AND METHODS

Reagents

Bovine RCM-κ-casein, was prepared according to Groves *et al*.[17] Commercial 225 and 75 Bloom gelatins, type B from bovine skin, characterized at this laboratory as 225 g and 95 g Bloom (G-225 and G-95), respectively, were obtained from Sigma (St Louis, MO, USA). Worsted flannel wool fabrics (Testfabrics Inc, No 523, balanced plain weave) were purchased from Testfabrics (West Pittston, PA, USA).

Activa TG-TI, a microbial transglutaminase (100 units g^{-1}) containing maltodextrin as a carrier, with activity in the pH 4.0–9.0 range, at 0–70 °C, was obtained from Ajinomoto USA Inc (Paramus, NJ, USA). *o*-Phosphorylethanolamine, Triton X-100, polyvinyl alcohol, *N*-ethylmaleimide, malachite green–HCl, ammonium molybdate and hydrogen peroxide were purchased from Sigma (St Louis, MO, USA). Phast gels, 4–15% gradient, were purchased from Pharmacia (Hercules, CA, USA). All other reagents used were of analytical grade or ACS certified.

Model system for transglutaminase-catalysed reaction on RCM-κ-casein

Monomeric RCM-κ-casein (2.8 mg) was suspended in 1.0 ml of 0.1 M Tris–HCl (pH 8.5); 0.5 ml of the resulting solution was pre-incubated at 37 °C for 1 h. Aliquots (250 µl) of the incubated RCM-κ-casein and non-pre-incubated controls were dispensed into small test tubes (two each). To one test tube containing the pre-incubated casein and to one tube containing the control were added 50 µl of a solution containing 14 µg ml^{-1} of TGase and 50 µl of 71 mg ml^{-1} *o*-phosphorylethanolamine, both dissolved in Tris-buffer. A control tube contained the same amount of *o*-phosphorylethanolamine with no enzyme. All tubes were incubated for 3 h at 37 °C followed by heating at 90 °C for 5 min to inactivate the enzyme. The efficiency of the reaction was determined as follows: half of the catalysed reaction was saved for SDS–PAGE analysis to check for the formation of crosslinks. The other half was dialysed to remove excess amine and treated as described below to quantitate the amount of bound phosphate.

Electrophoretic analysis

SDS–PAGE was run on a Pharmacia Phast Gel system according to the method of Laemmli.[18] Samples were loaded on 4–15% gradient gels. After electrophoresis the gels were stained with Coomassie brilliant blue R-250 and destained. A flow chart of the samples run by the above method is shown in Fig 2.

Determination of phosphate bonded to κ-casein

Dialysed phosphorylated RCM-κ-Casein (100 µl of 2.4 mg ml^{-1}) was freeze-dried. HCl (600 µl of 6 M) was added to the dry flask which then was flushed with nitrogen and sealed. The treated tubes were hydrolyzed overnight at 113 °C. Analysis of the inorganic phosphate released was carried out using the malachite green assay[19] and read at 630 nm using a Varian Cary-50 spectrophotometer to detect the presence of inorganic phosphate.

Model system for transglutaminase-catalysed reaction on gelatin

Gelatins are classified by their Bloom value, a measure of their strength. Stronger gels give higher Bloom values. G-95 and G-225 (20 mg each) gelatins were suspended in 2 ml (0.1 M) Tris–HCl pH 8.5 and allowed to swell for 1 h. The resulting suspensions were heated to 60 °C for a few minutes for the particles to dissolve and then were divided into four 500 µl aliquots. To tube 1 was added 50 µl of 14 µg ml^{-1} of TGase and the primary amine *o*-phosphorylethanolamine (50 µl of 71 mg ml^{-1}); to tube 2, 50 µl of 14 µg ml^{-1} of TGase; to tube 3 the primary amine *o*-phosphorylethanolamine (50 µl of 71 mg ml^{-1}); and to the control tube 4 no additions.

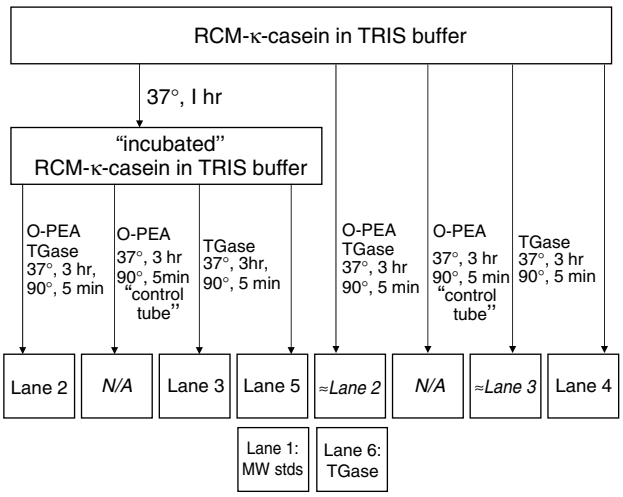

Figure 2. Variations in reaction conditions and correlation to SDS–PAGE gel lanes (Fig 3). N/A, not applicable; O-PEA, *o*-phosphorylethanolamine.

All the tubes were incubated at 40 °C for 4 h. The reaction was terminated by the addition of 1% *N*-ethylmaleimide solution. The samples were dialysed, and the bound inorganic phosphate was detected by hydrolysis and malachite green assay[19] as mentioned above. The efficiency of the reaction was also detected by SDS–PAGE analysis.

Transglutaminase-catalysed reaction on wool fabric

Four pieces of woven wool fabric, approximately 1.5 g each, were used. Fabrics were placed in individual reaction tubes containing 100 ml of 0.1 M Tris–HCl buffer, pH 8.5 and 1% Triton X-100 for 1 h before the addition of 0.015 g TGase dissolved in Tris–HCl buffer. Four concentrations of *o*-phosphorylethanolamine (35, 71, 106 and 142 mM) were used. The tubes were incubated at 40 °C for 4 h. The reaction was terminated with 1 ml of 1% *N*-ethylmaleimide solution. The fabric was rinsed five times with distilled water and vacuum-dried before further analysis. Bound phosphate was analysed using the method described below. The fabrics were also subjected to mechanical testing as described below.

Determination of phosphate in wool fabric

Approximately 0.4 g samples of phosphorylated woven wool fabric from the above experiment were each placed into a Kjeldahl flask. The fabric was digested with 1 ml of 50% sulphuric acid (v/v) and heated until the solution darkened.[20] The flask was cooled for 15 s before a few drops of 30% hydrogen peroxide were added. The mixture was again allowed to simmer for 20 min. The cycle was repeated several times with the addition of two to four drops of hydrogen peroxide until solutions turned pale yellow or colourless. Phosphate analysis on the woven wool fibre was performed as reported.[20]

Tensile strength

Mechanical property measurements were performed on the wool fabric. Measurements included tensile strength, Young's modulus and initial strain energy. Tensile strength is the maximum stress sustained under a tensile force without fracture.[21] Young's modulus is a physical quantity representing the stiffness of a material. It is determined by measuring the slope of a line tangent to the initial stress–strain curve. The initial strain energy is defined as the energy needed to stretch the fabric to 10% strain.[22] This is the area under the stress–strain curve from 0 to 10% strain. If other material variables are equal, the initial strain energy will be proportional to the volume of the tested samples. To compare different samples, the value of initial strain energy for each test sample was divided by the volume of that sample to obtain the initial strain energy with the SI unit of $J\,cm^{-3}$. These properties were measured with a gauge length (the distance between two grips) of 25.4 mm. Test samples were stored in a conditioned room at 23 °C and 65% RH before testing according to ASTM standard method D1610-01. An upgraded Instron mechanical property tester, model 1122 and Testworks 3.1 data acquisition software (MTS Systems Corp, Minneapolis, MN, USA) were used throughout this work. The strain rate (crosshead speed) was set at $300\,mm\,min^{-1}$. Because of limited sample availability, each mechanical property test was run in sets of five.

RESULTS AND DISCUSSION

Transglutaminase-catalysed reaction on κ-casein

The caseins of milk form a unique calcium-phosphate transport complex, which provides necessary nutrients to the neonate. The colloidal stability of these particles is due primarily to κ-casein. As purified from milk, this protein exhibits a unique disulphide bonding pattern, which (in the absence of reducing agents) ranges from monomer to octamers and above on SDS–PAGE. Recent studies of κ-casein have shown that, when the protein was reduced, carboxymethylated (RCM-κ-casein) and pre-incubated (tempered) for 30 min at 37 °C, the protein formed fibrillar structures instead of spherical particles.[23,24]

The fibrillar structures were up to 400 nm in length. Circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopies had been used in this laboratory to investigate the temperature-induced changes in the secondary structure of the RCM-κ-casein. These studies had suggested little change in the distribution of secondary structural elements during this transition, with extended strand and β turns predominating, and previous studies on amyloid proteins have suggested that such motifs promote fibril formation.[25]

Christensen *et al*[26] studied the peptides resulting from the reaction of TGase with κ-casein in the presence of the diamine putrescine. They demonstrated that Gln residues 29, 45, 114 and 163 were good acyl donors for the reaction with putrescine. Additionally, Lys residues 21 and 24 formed either inter- or intra-molecular cross-linkages with Gln 45. It is important to note that in their studies the κ-casein was thoroughly reduced with DTT prior to incubation with the TGase at 37 °C for 3 h. The conditions used by Christensen and coworkers would have initiated the formation of fibrils noted above. The physical aggregation to fibrils could be the cause of inter-molecular cross-linkages.

In our studies, RCM-κ-casein was used to evaluate the ability of TGase to cross-link the protein. The lack of disulphide bonds in the modified protein causes it to migrate as low molecular weight species on SDS–PAGE, so the presence of cross-links induced by TGase could readily be evaluated by this method. Figure 3 shows the un-reacted enzyme in lane 6 and the non-pre-incubated RCM-κ-casein in lane 4; both migrate as essentially single bands with reduced molecular weights of 33 000 and 19 000, respectively.

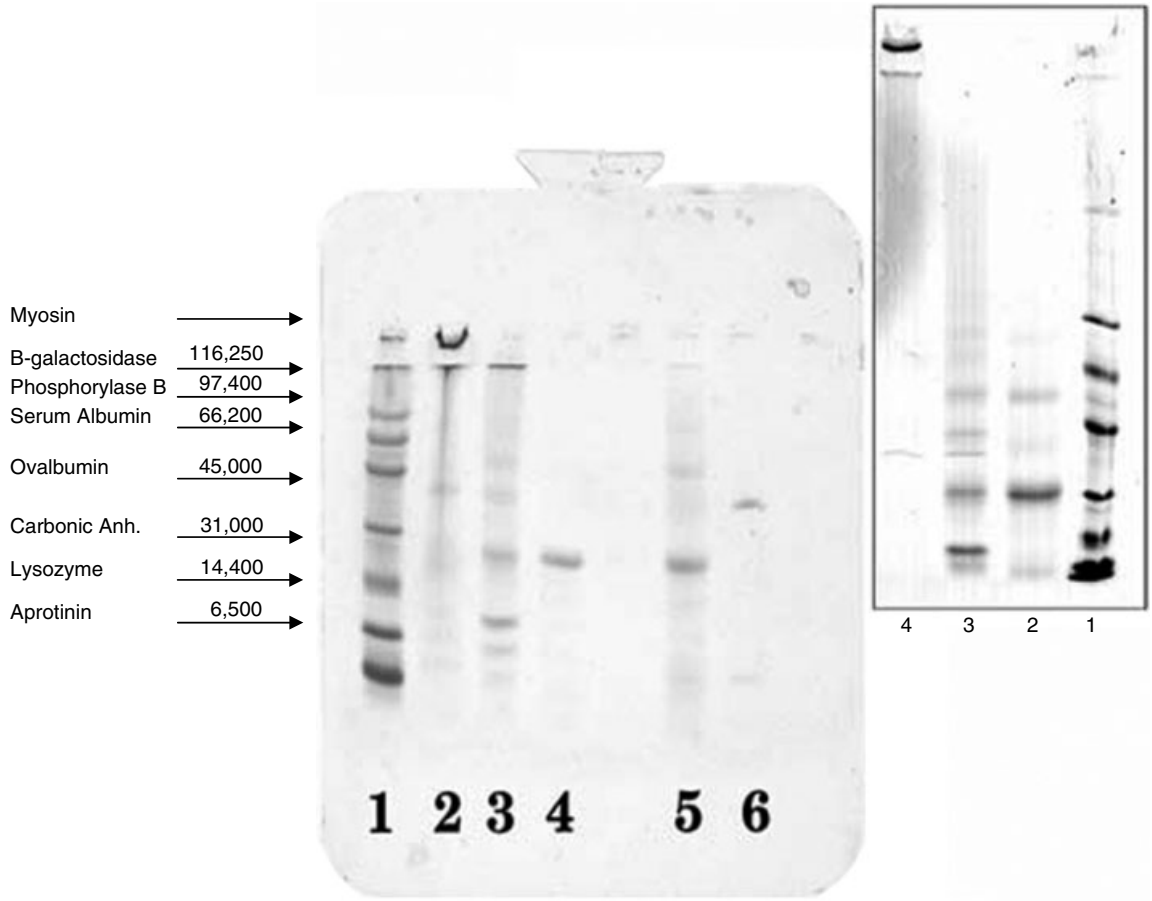

Figure 3. SDS–PAGE gel electrophoresis showing cross-linkage in RCM-κ-casein and covalent bonding with *o*-phosphorylethanolamine (O-PEA). Lane 1 shows molecular weight standards; lane 2 shows pre-incubated RCM-κ-casein, TGase and *o*-phosphorylethanolamine; lane 3 shows pre-incubated RCM-κ-casein with TGase enzyme; lane 4 shows non-pre-incubated RCM-κ-casein; lane 5 shows pre-incubated RCM-κ-casein without the enzyme TGase; lane 6 shows TGase alone. (Inset shows the molecular weight standards (lane 1) and non-preincubated RCM-κ-casein (lane 2), with enzyme TGase (lane 3) and enzyme TGase plus O-PEA (lane 4).).

Lane 5 shows pre-incubated RCM-κ-casein in the absence of any reactants; small amounts of SDS-resistant dimer and trimer are caused by the incubation at 37 °C. The reaction of RCM-κ-casein at 37 °C with the enzyme TGase alone (lane 3) causes a significant increase in higher-molecular-weight species, including polymers of >100 000, which are unable to penetrate the separating portion of the SDS–PAGE gel; these conditions are equivalent to those of Christensen *et al*,[26] who demonstrated both amine incorporation and cross-linkages. The reaction products of TGase with RCM-κ-casein that was not pre-incubated at 37 °C to promote fibril formation were identical to those seen in lane 3 (Fig 3). In this preparation of TGase, some protease may also be present, as lower molecular weight species of RCM-κ-casein are also present (lane 3). In contrast, the presence of *o*-phosphorylethanolamine in the pre-incubated reaction mixture as shown in lane 2 (and the non-preincubated reaction mixture; Fig 3 inset) led to the formation of highly cross-linked polymeric species, which, for the most part, are unable to penetrate even the low porosity SDS stacking gel (lane 2). Apparently the *o*-phosphorylethanolamine not only may be attached to the RCM-κ-casein, but also somehow leads to either increased inter-molecular cross linkages that are not DTT-sensitive, or increased protein–protein interactions that are not broken down by SDS. Figure 4 shows the amount of phosphate bound to the RCM-κ-casein.

Transglutaminase-catalysed reaction with gelatin

As reported,[2] the physical properties of gelatin can be modified with the use of enzyme under certain

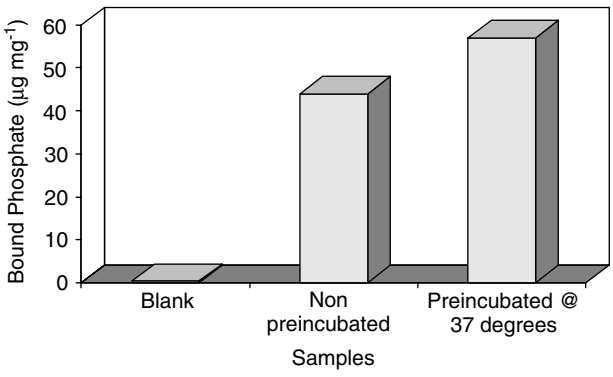

Figure 4. Amount of phosphate bound to untreated and treated RCM-κ-casein.

experimental conditions. When one to five units of the enzyme were used per gram of gelatin, the melting point and the Bloom strength increased. In the present work, G-225 and G-95 gelatin samples were reacted as described in the Materials and Methods. Although gelatins are blends of varying molecular weights and do not produce well defined bands on SDS–PAGE gels, Fig 5 shows that, in the presence of TGase, intra- and inter-molecular crosslinks led to the formation of large aggregates that could not enter the SDS–PAGE gel. Lanes that contain the enzyme or both the enzyme and *o*-phosphorylethanolamine demonstrated high-molecular-weight polymers, as the protein did not migrate through the low porosity stacking gel. Samples containing gelatin and *o*-phosphorylethanolamine alone showed no difference from the control sample (gelatin alone), showing that, if any crosslinking had occurred, it must have been minimal as it was not detected on the gel (not shown). These samples were later dialysed to remove unbound phosphate and analysed for bound phosphate using the malachite green assay (Fig 6). G-95g with *o*-phosphorylethanolamine without TGase was used as a control to confirm complete dialysis of unbound *o*-phosphorylethanolamine.

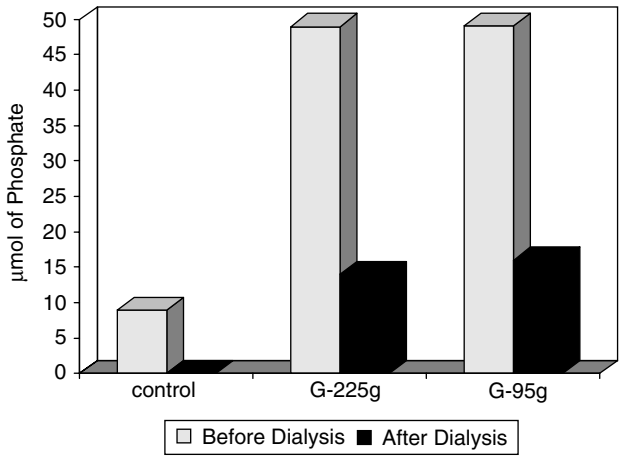

Figure 6. Amount of phosphate bound to gelatin (using malachite green assay[23]) after dialysis to release unbound phosphate. Control: G-95g gelatin plus *o*-phosphorylethanolamine, no TGase; G-225g and G-95g: gelatins plus *o*-phosphorylethanolamine plus TGase.

Transglutaminase-catalysed reaction on wool fabric

We investigated the covalent binding of a primary amine to wool through a TGase-catalysed reaction. Wool fabric was treated with TGase as described in the materials and Methods. After several washings, approximately 0.4 g of the phosphorylated wool fabric was digested with acid and analysed for bound phosphate using the EPA method.[20] These results indicate that, after several washings, some *o*-phosphorylethanolamine is covalently bonded to the wool fibre/fabric. The data, as shown in Fig 7, indicate that *o*-phosphorylethanolamine is covalently bonded

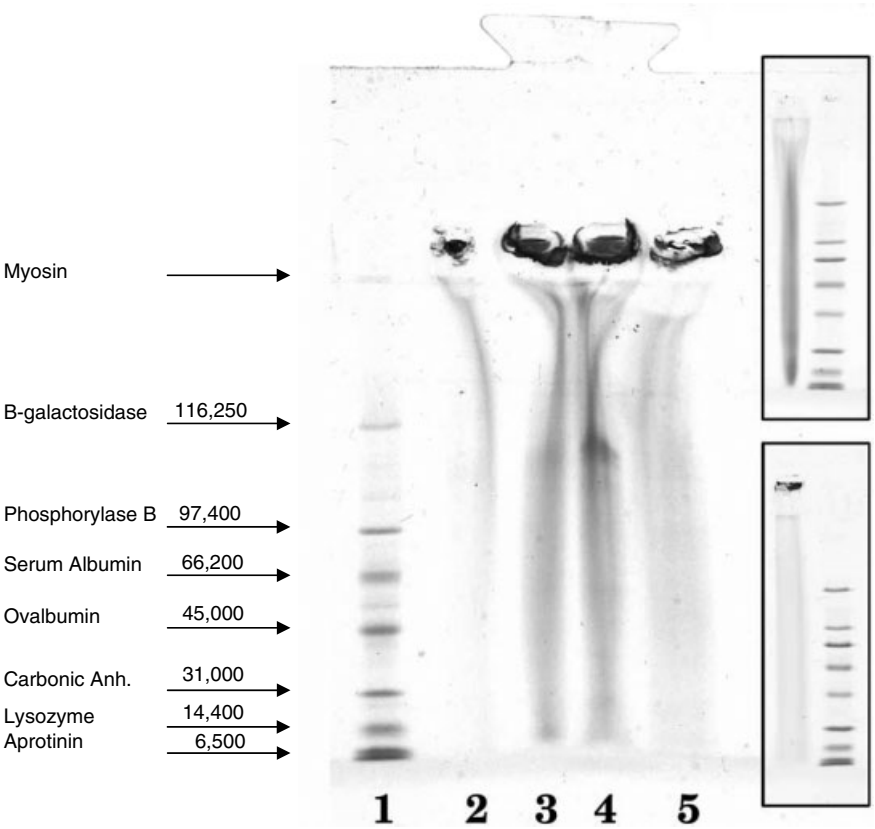

Figure 5. SDS–PAGE showing the cross-linkage and covalent bonding with gelatin. Lane 1 shows molecular weight standards; lane 2 shows G-225g with TGase; lane 3 shows G-225g with TGase and *o*-phosphorylethanolamine; lane 4 shows G-95g with the enzyme TGase; lane 5 shows G-95g with the enzyme TGase and *o*-phosphorylethanolamine. (Insets show molecular weight standards and gelatin with two different Bloom values: top inset G-225g and lower inset G-95g.).

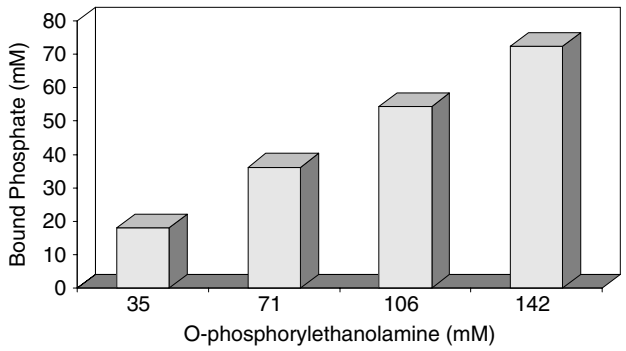

Figure 7. Amount of phosphate bound to wool fiber using EPA method 365.3[24] as a function of *o*-phosphorylethanolamine charge (35, 71, 106 and 142 mM, respectively) in the reaction with 0.4 g wool.

to the wool fibre/fabric since it survived four washings. For the wool fibres, the reaction is concentration-dependent, with a final concentration of 142 mM of *o*-phosphorylethanolamine being most effective (Fig 7).

Tensile strength

Tensile strength can be defined as the measure of a steady force that is necessary to break a fibre and it is usually expressed experimentally as the maximum load developed in tensile test.[26] There are many different ways of reporting breakage resistance of fabrics, such as by force, elongation and energy necessary. In Fig 8 are the tensile strength measurements of the *o*-phosphorylethanolamine-treated fabric before and after treatments. The tensile strength of the control fabric (no TGase, and *o*-phosphorylethanolamine) overall did not show a major change with the increase of *o*-phosphorylethanolamine in the two samples used. However, in Fig 9, the elongation property increased with increased levels of *o*-phosphorylethanolamine. The enzymatic addition of *o*-phosphorylethanolamine did not affect the tensile strength of the fabric, but the elongation property was dramatically increased.

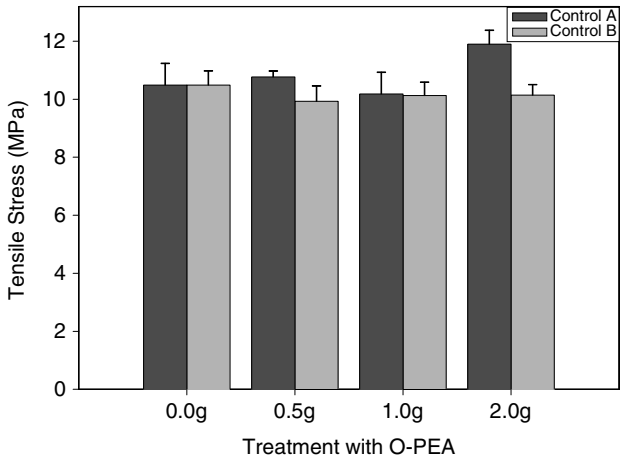

Figure 8. Tensile strength of woven fabric with TGase and *o*-phosphorylethanolamine.

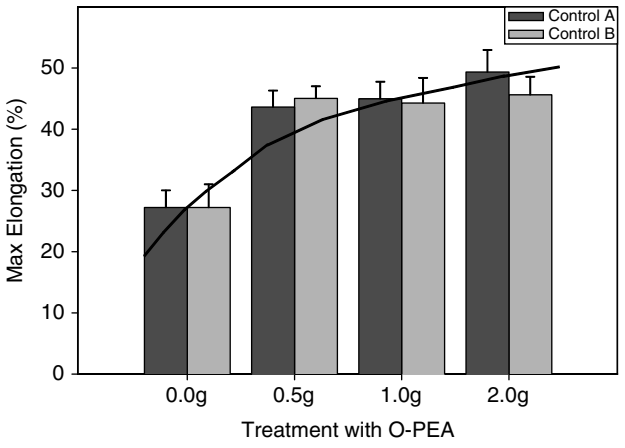

Figure 9. Elongation property of woven fabric.

CONCLUSIONS

In this study, we examined TGase-catalysed crosslinking and covalent bonding on two soluble proteins and on wool fiber. We provided evidence that TGase produced inter- and intra-molecular crosslinks in the proteins used and also catalysed the covalent bonding of *o*-phosphorylethanolamine to RCM-κ-casein, gelatin and wool. The preliminary results using TGase with the fibre are encouraging as the treated wool was whiter with a softer feel than the untreated fibre. We are conducting further studies to determine the treatment effect on wool's mechanical and flame-retardant properties and also on the tensile strength of the treated fibres.

ACKNOWLEDGEMENTS

We wish to thank Robert Dudley, Edwin Piotrowski, Nicholas Latona and Lorelei Bumanlag for their technical support in this work.

REFERENCES

1 Hemmpel WH, The surface modification of woven and knitted cellulose fiber fabric by enzymatic degradation. *Int Tex Bull Dye Print Finish* **3**:5–8 (1991).
2 Taylor MM, Liu CK, Latona N, Marmer WN and Brown EM, Enzymatic modification of hydrolysis products from collagen using a microbial transglutaminase. Preparation of films. *J Amer Leather Chem Assoc* **97**:225–234 (2002).
3 Chikkodi SV, Khan S and Mehta RD, Effects of biofinishing on cotton/wool blended fabrics. *Tex Res J* **65**:564–569 (1995).
4 Zaks A, Industrial biocatalysis. *Current Opinion in Chem Biol* **5**:130–136 (2001).
5 Heine E and Höcker H, Enzyme treatment for wool and cotton. *Rev Prog Coloration* **25**:57–63 (1995).
6 Schmid A, Hollmann F, Park JB and Buhler B, The use of enzymes in chemical industry in Europe. *Curr Opin Biotechnol* **13**:359–366 (2002).
7 Lorand L, Konishi K and Jacobsen A, Transpeptidation mechanism in blood clotting. *Nature* **194**:1148–1149 (1962).
8 Folk JE, Transglutaminases. *A Rev Biochem* **49**:517–531 (1980).
9 Ichinose A, Bottenus RE and Davie EW, Structure of transglutaminases. *J Biol Chem* **265**:13 411–13 414 (1990).
10 Ikura K, Kometani K, Yoshikawa M, Sasaki R and Chiba H, Crosslinking of casein components by transglutaminase. *Agric Biol Chem* **44**:1567–1573 (1980).

11 Motoki M and Nio N, Cross-linking between different food proteins by transglutaminase. *J Food Sci* **48**:561–566 (1983).
12 Folk JE, Mechanism and basis for specificity of transglutaminase catalyzed ε-(γ-glutamyl) lysine bond formation. *Adv Enzymol* **54**:1–56 (1983).
13 Nielsen PM, Reactions and potential industry applications of transglutaminase. Review of literature and patents. *Food Biotech* **9**:119–156 (1995).
14 McDevitt JP and Winkler J, Method for enzymatic treatment of wool. United States Patent 6,051,033 (2000).
15 Colas B, Caer D and Fournier E. Transglutaminase catalyzed glycosylation of vegetable proteins. Effect on solubility of pea legumin and wheat gliadins. *J Agric Food Chem* **41**:1811–1815 (1993).
16 Gorman JJ and Folk JE. Structural features of glutamine substrates for human plasma factor XIIIa (activated blood coagulation factor XIII). *J Biol Chem* **255**:419–427 (1980).
17 Groves ML, Wickham ED and Farrell HM Jr, Environmental effects on disulfide bonding patterns of bovine κ-casein. *J Prot Chem* **17**:73–84 (1998).
18 Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227**:680–685 (1970).
19 Chan KM, Delfert D and Junger KD. A direct colorimetric assay for Ca^{2+} stimulated ATPase activity. *Anal Biochem* **157**:375–380 (1986).
20 Environmental Protection Agency, *Methods for Chemical Analysis of Water and Waste. Method 365.3*. Office of Research and Development, Cincinnati, OH (1983).
21 Liu C-K, and McClintick MD, Measurements of the initial strain energy of leather. *JALCA* **92**:157–171 (1997).
22 Morton WE and Hearle JWS, *Physical Properties of Textile Fibers*, 2nd edn. J Wiley, New York (1975).
23 Farrell HM Jr, Qi PX, Brown EM and Cooke PH, Molten globule structures in milk proteins: Implications for potential new structure-function relationships. *J Dairy Sci* **85**:459–471 (2002).
24 Farrell HM Jr, Cooke PH, Wickham ED, Piotrowski EG and Hoagland PD. Environmental influences on bovine κ-casein: reduction and conversion to fibrillar (amyloid) structures. *J Prot Chem* **22**:259–273 (2003).
25 Wetzel R, Domain stability in immunoglobulin light chain deposition disorders. *Adv Prot Chem* **50**:183–242 (1997).
26 Christensen BM, Sorensen ES, Hojrup P, Petersen TE and Rasmussen LK, Localization of potential transglutaminase cross-linking sites in bovine caseins. *J Agric Food Chem* **44**:1943–1947 (1996).